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Filed: 4 May 2001
For: AFFINITY SELECTED SIGNATURE PEPTIDES FOR PROTEIN IDENTIFICATION AND
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Amendments to the Claims

This listing of claims replaces all prior versions, and listings, of claims in the above-identified application:

Listing of Claims

1. **(original)** A method for detecting a difference in the concentration of a protein present in a first sample and in a second sample, each sample comprising a plurality of proteins, the method comprising:

covalently attaching a first isotopic variant of a chemical moiety to a protein in the first sample to yield at least one first isotopically labeled protein;

covalently attaching a second isotopic variant of the chemical moiety to a protein in the second sample to yield at least one second isotopically labeled protein, wherein the first and second isotopically labeled proteins are chemically equivalent yet isotopically distinct;

mixing at least portions of the first and second samples to yield a combined sample; and

subjecting the combined sample to mass spectrometric analysis to determine a normalized isotope ratio characterizing proteins whose concentration is the same in the first and second samples and an isotope ratio of the first and second isotopically labeled proteins, wherein a difference in the isotope ratio of the first and second isotopically labeled proteins and the normalized isotope ratio is indicative of a difference in concentration of the protein in the first and second samples.

2. **(original)** The method of claim 1 further comprising fractionating the combined sample to yield at least one fraction comprising the isotopically labeled first and second proteins prior to determining the isotope ratios.

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3. **(original)** The method of claim 2 wherein fractionating the combined sample comprises subjecting the proteins to multidimensional chromatography, two-dimensional electrophoresis, affinity fractionation, or a combination thereof.

4. **(original)** A method for detecting a difference in the concentration of a protein present in a first sample and in a second sample, each sample comprising a plurality of proteins, the method comprising:

covalently attaching a first isotopic variant of a chemical moiety to a protein in the first sample to yield at least one first isotopically labeled protein;

covalently attaching a second isotopic variant of the chemical moiety to a protein in the second sample to yield at least one second isotopically labeled protein, wherein the first and second isotopically labeled proteins are chemically equivalent yet isotopically distinct;

fragmenting proteins in the first and second samples to yield first and second isotopically labeled peptides in the first and second samples, respectively;

mixing at least portions of the first and second samples to yield a combined sample, wherein mixing is performed before or after fragmentation; and

subjecting the combined sample to mass spectrometric analysis to determine a normalized isotope ratio characterizing peptides derived from proteins whose concentration is the same in the first and second samples and an isotope ratio of the first and second isotopically labeled peptides, wherein a difference in the isotope ratio of the first and second isotopically labeled peptides and the normalized isotope ratio is indicative of a difference in concentration in the first and second samples of a protein derived from the peptide.

5. **(original)** A method for detecting a difference in the concentration of a protein present in a first sample and in a second sample, each sample comprising a plurality of proteins, the method comprising:

fragmenting proteins in the first and second samples to yield at least one peptide in each

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sample;

covalently attaching a first isotopic variant of a chemical moiety to a peptide in the first

sample to yield at least one first isotopically labeled peptide;

covalently attaching a second isotopic variant of the chemical moiety to a peptide in the

second sample to yield at least one second isotopically labeled peptide, wherein the first and

second isotopically labeled peptides are chemically equivalent yet isotopically distinct;

mixing at least portions of the first and second samples to yield a combined sample; and

subjecting the combined sample to mass spectrometric analysis to determine a

normalized isotope ratio characterizing peptides derived from proteins whose concentration is

the same in the first and second samples and an isotope ratio of the first and second isotopically

labeled peptides, wherein a difference in the isotope ratio of the first and second isotopically

labeled peptides and the normalized isotope ratio is indicative of a difference in concentration in

the first and second samples of a protein derived from the peptide.

6. (currently amended) A method for detecting a difference in the concentration of a protein

originally present in a first sample and in a second sample, each sample comprising a plurality of

peptides derived from fragmentation of proteins originally present in the sample, the method

comprising:

covalently attaching a first isotopic variant of a chemical moiety to a peptide in the first

sample to yield at least one first isotopically labeled peptide;

covalently attaching a second isotopic variant of the chemical moiety to a peptide in the

second sample to yield at least one second isotopically labeled peptide, wherein the first and

second isotopically labeled peptides are chemically equivalent yet isotopically distinct;

mixing at least portions of the first and second samples to yield a combined sample; and

subjecting the combined sample to mass spectrometric analysis to determine a

normalized isotope ratio characterizing peptides derived from proteins whose concentration is

the same in the first and second samples and an isotope ratio of the first and second isotopically

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labeled peptides, wherein a difference in the isotope ratio of the first and second isotopically labeled peptides and the normalized isotope ratio is indicative of a difference in concentration in the first and second samples of a protein derived from the peptide.

7. **(original)** The method of claim 6 wherein the first and second chemical moieties are attached to at least one amino group on peptides in the first and second samples.

8. **(original)** The method of claim 6 wherein each member of at least one pair of chemically equivalent, isotopically distinct peptides comprises at least one affinity ligand, the method further comprising, prior to determining the isotope ratios, contacting the peptides with a capture moiety to select peptides comprising the at least one affinity ligand.

9. **(original)** The method of claim 8 further comprising subjecting the selected peptides comprising the at least one affinity ligand to mass spectrometric analysis to detect at least one peptide; and identifying the protein from which the detected peptide was derived.

10. **(original)** The method of claim 9 wherein the detected peptide is a signature peptide for a protein, the method further comprising determining the mass of the signature peptide and using the mass of the signature peptide to identify the protein from which the detected peptide was derived.

11. **(original)** The method of claim 10 further comprising determining the amino acid sequence of the detected peptide and using the amino acid sequence of the detected peptide to identify the protein from which the detected peptide was derived.

12. **(original)** The method of claim 8 further comprising subjecting the selected peptides

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comprising the at least one affinity ligand to mass spectrometric analysis to determine peak intensities; and quantitating isotope ratios from the peak intensities.

13. **(original)** The method of claim 8 further comprising, prior to contacting the peptides with the capture moiety, covalently attaching at least one affinity ligand to at least one peptide derived from the fragmentation of the proteins.

14. **(original)** The method of claim 5 further comprising, prior to fragmenting the proteins, covalently attaching at least one affinity ligand to at least one protein in the sample.

15. **(original)** The method of claim 5 further comprising reducing and alkylating the proteins with an alkylating agent prior to fragmenting the proteins.

16. **(original)** The method of claim 15 wherein the at least one affinity ligand is covalently attached to the alkylating agent.

17. **(original)** The method of claim 8 wherein the at least one affinity ligand is covalently attached to an amino acid of the peptide selected from the group consisting of cysteine, tyrosine, tryptophan, histidine and methionine.

18. **(original)** The method of claim 8 wherein the affinity ligand comprises a moiety selected from the group consisting of a peptide antigen, a polyhistidine, a biotin, a dinitrophenol, an oligonucleotide and a peptide nucleic acid.

19. **(original)** The method of claim 8 wherein at least one peptide comprises an endogenous affinity ligand.

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20. **(original)** The method of claim 19 wherein the endogenous affinity ligand comprises a moiety selected from the group consisting of a cysteine, a histidine, a phosphate group, a carbohydrate moiety and an antigenic amino acid sequence.

21. **(original)** The method of claim 10 comprising attaching a plurality of affinity ligands, each to at least one protein or peptide, and contacting the peptides with a plurality of capture moieties to select peptides comprising at least one affinity ligand.

22. **(original)** The method of claim 5 wherein the proteins are fragmented using an enzyme selected from the group consisting of trypsin, chymotrypsin, gluc-C, endo lys-C, pepsin, papain, proteinase K, carboxypeptidase, calpain and subtilisin.

23. **(original)** The method of claim 6 further comprising fractionating the peptides prior to determining the isotope ratios.

24. **(original)** The method of claim 23 wherein fractionating the peptides comprises subjecting the peptides to at least one separation technique selected from the group consisting of reversed phase chromatography, ion exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography, capillary gel electrophoresis, capillary zone electrophoresis, and capillary electrochromatography, capillary isoelectric focusing, immobilized metal affinity chromatography and affinity electrophoresis.

25. **(original)** The method of claim 6 wherein the sample comprises at least about 100 proteins.

26. **(original)** The method of claim 10 wherein using the mass of the signature peptide to identify the protein from which the signature peptide was derived comprises comparing the mass of the signature peptide with the masses of reference peptides derived from putative proteolytic

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cleavage of a plurality of reference proteins in a database, wherein at least one reference peptide
comprises at least one affinity ligand.

27. **(original)** The method of claim 26 wherein peptides derived from proteolytic cleavage of
the plurality of reference proteins are, prior to comparing the mass of the signature peptide with
the masses of the reference peptides, computationally selected to exclude reference peptides that
do not contain an amino acid upon which the affinity selection is based.

28. **(original)** The method of claim 6 wherein the protein is in regulatory flux in response to a
stimulus, and wherein the first sample is obtained from the biological environment before
application of the stimulus and the second sample is obtained from the biological environment
after application of the stimulus.

29. **(original)** The method of claims 6 wherein the first and second samples are obtained from
different organisms, cells, organs, tissues or bodily fluids, the method further comprising
determining differences in concentration of at least one protein in the organisms, cells, organs,
tissues or bodily fluids from which the samples were obtained.

30. **(original)** The method of claim 1 further comprising identifying a plurality of isotopically
labeled proteins having substantially the same isotope ratios, wherein the existence of said
plurality of isotopically labeled proteins is indicative that the proteins are co-regulated.

31. **(original)** The method of claims 6 further comprising identifying a plurality of isotopically
labeled peptides having substantially the same isotope ratios, wherein the existence of said
plurality of isotopically labeled peptides is indicative that the peptides are derived from the same
protein, or from proteins that are co-regulated.

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32. **(original)** The method of claims 6 wherein the samples are obtained from a biological environment, and wherein the first sample is obtained from the biological environment before application of a stimulus and the second sample is obtained from the biological environment after application of the stimulus.

33.- 99. **(cancelled)**